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*Institute for  
Marine Biosciences*

# **From sequence to sickness: Using genomics and biotechnology to understand aquatic animal pathogens**

**Laura L. Brown**

**Bacterial Kidney Disease – Challenge for the 21<sup>st</sup> Century**

*Seattle, WA, USA, November 15-17, 2005*



National Research  
Council Canada

Conseil national  
de recherches Canada

**Canada**

**NRC-CNRC**

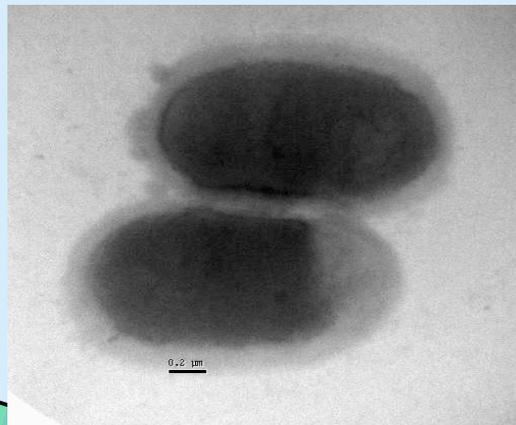
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# NRC's Genomics and Health Initiative: GHI



# *Aeromonas salmonicida* subsp. *salmonicida* A449

- Typical, non-motile *Aeromonas salmonicida* subsp. *salmonicida*
- Isolated from an infected brown trout in Eure, France by C. Michel in 1975
- Virulent, A-layer positive, LPS positive, hemolysin positive, cytotoxicity positive, elastase positive, protease negative, lecithin negative
- Chloramphenicol resistant
- Forms biofilms
- Upper temperature limit of  $\sim 30^{\circ}\text{C}$



# Genome Overview

## Chromosome

4,702,400 bp

58.51% G+C

4429 genes (566 unique)

10 types of insertion sequences

2 prophages

9 rRNA operons

110 tRNAs

## 7 Plasmids

5247 bp (8)

5424 bp (7)

5616 bp (9)

15966 bp (20)

111161 bp (140)

166749 bp (194)

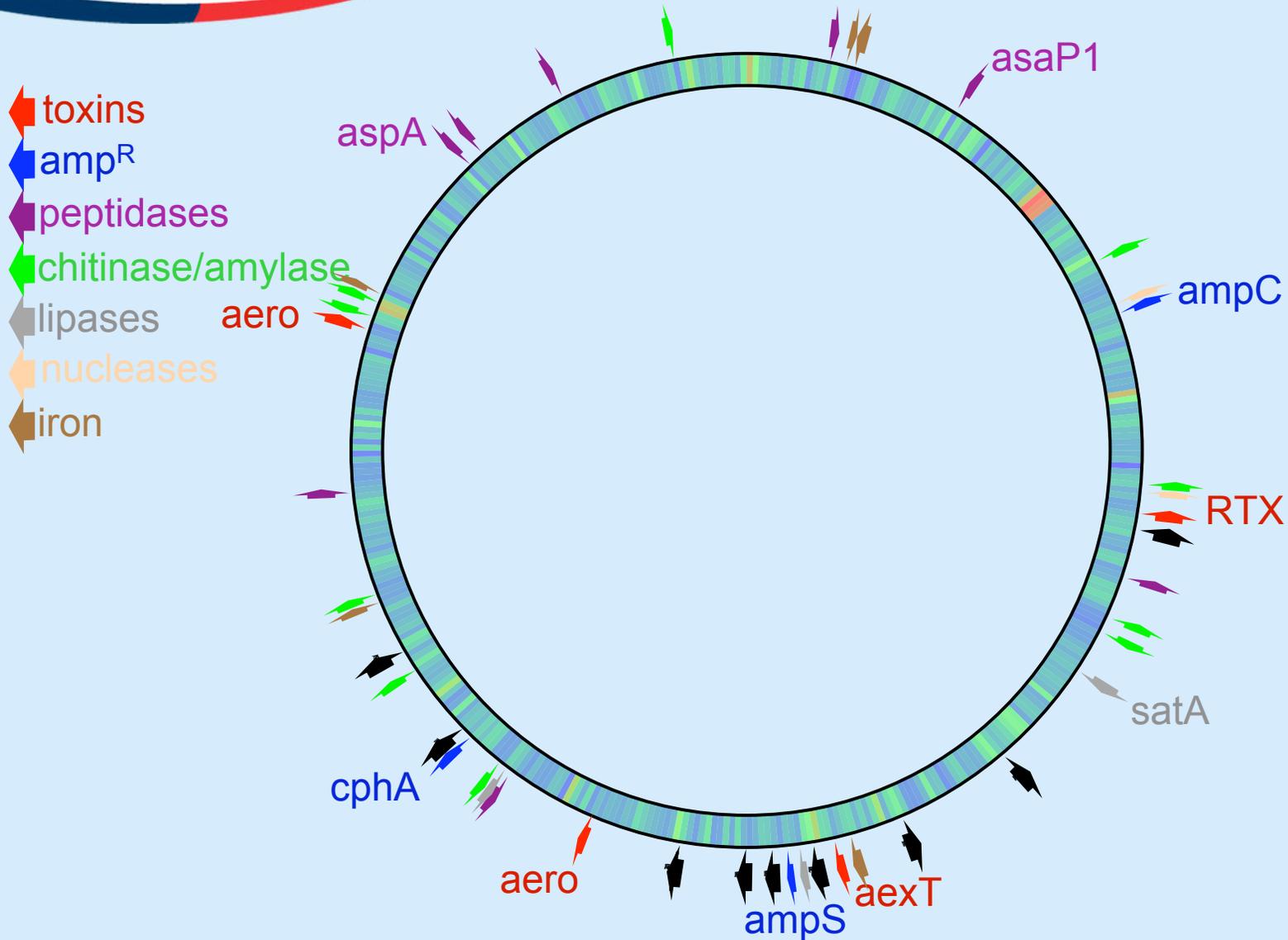
>32400 bp (>31)

Total Genome >5,045,000 bp

Genes >4838

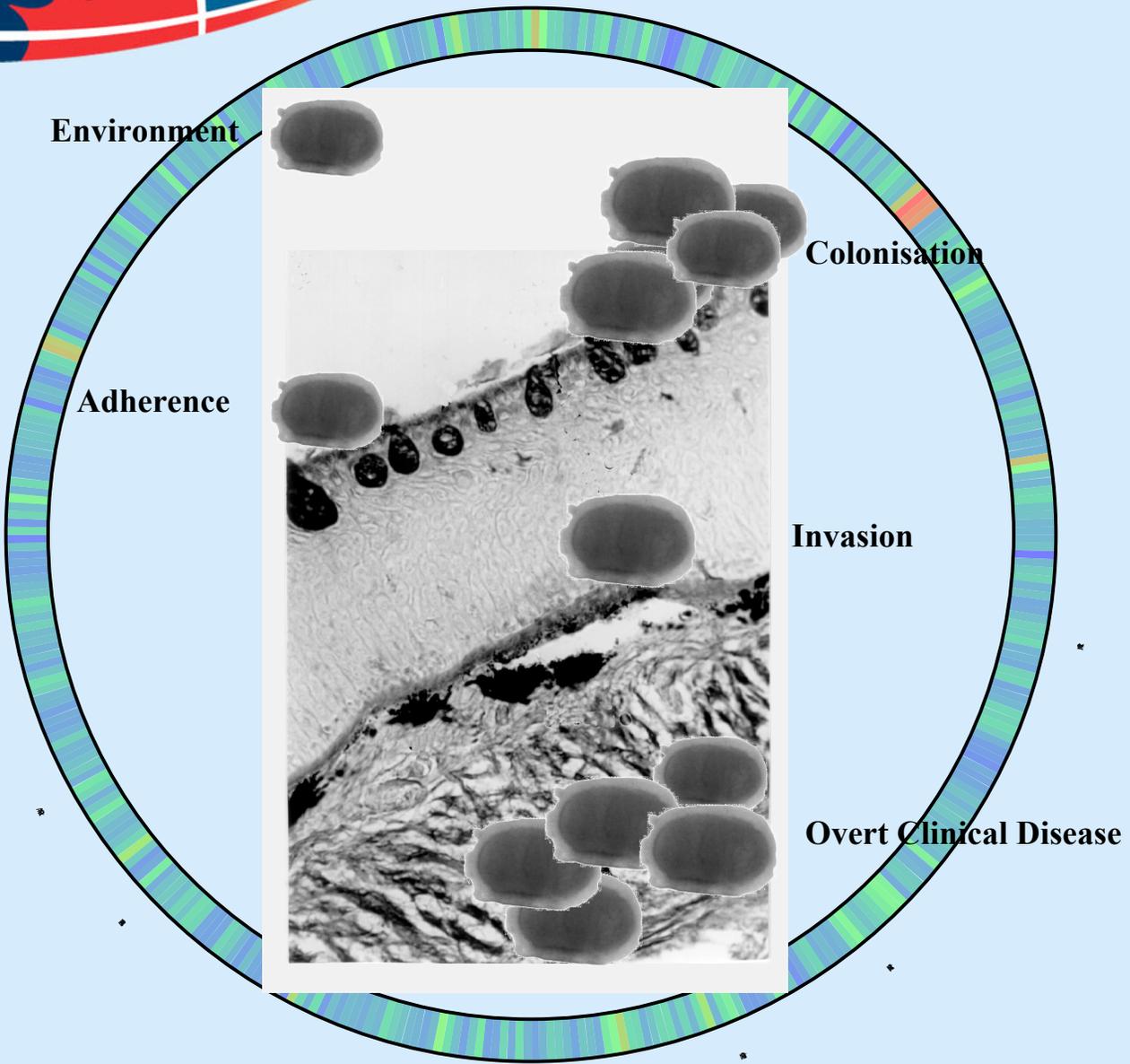
Unique Genes > 696 (~14%)

# Virulence factors – toxins, enzymes, etc.



# Summary

- The genome contains a large number of virulence factors that can be identified by homology to known genes, although there are undoubtedly more that haven't yet been recognized
- Motility genes have been maintained and conserved in an ostensibly non-motile organism
- *A. salmonicida* has a number of features that distinguish it from other *Aeromonas* species
- Genomic sequence comparisons between *Aeromonas* species will be a useful first step for identifying additional virulence genes as well as for identifying markers for taxonomic and epidemiological analysis



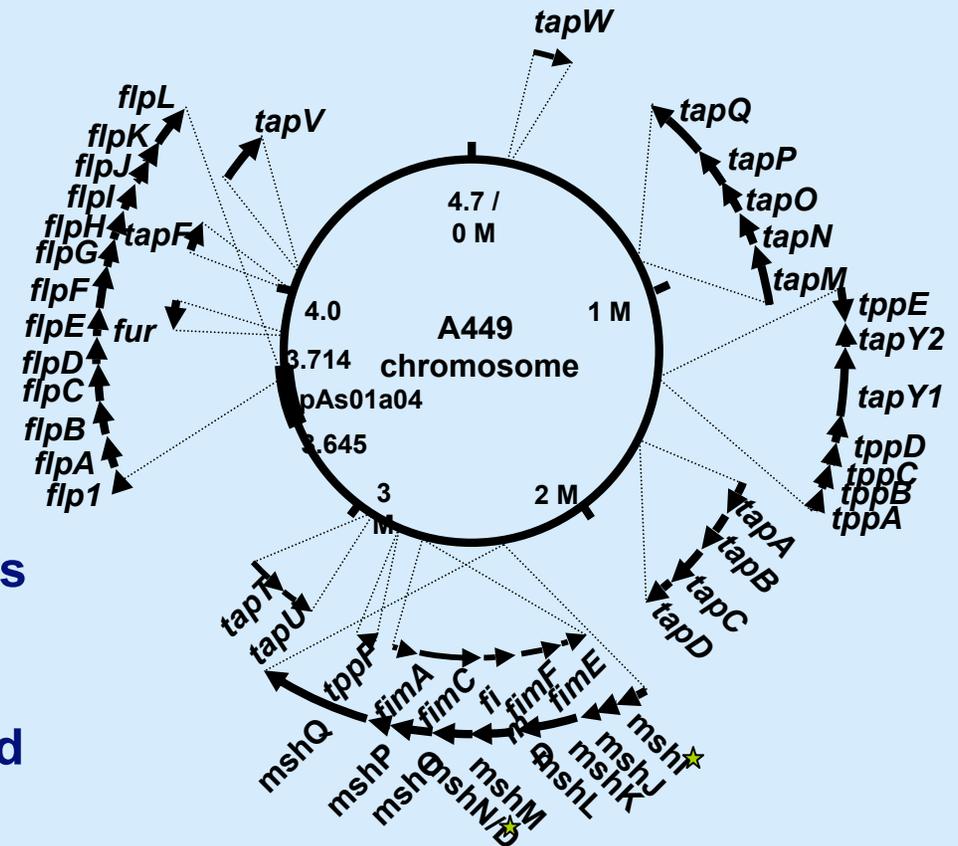
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# Adhesins

- **S-layer**
  - Major known adhesin & virulence factor
    - S-layer usually correlates with virulence
- **Type IV pili**
  - Petrichous or polar
  - Well characterised adhesins
    - *Pseudomonas aeruginosa*
    - *Neisseria* spp
  - Three pili operons identified
    - *MSHA*
    - *Flp*
    - *Tap*



# Dominance of S-layer in *in vitro* adhesion

- Pili and S-layer deficient strains

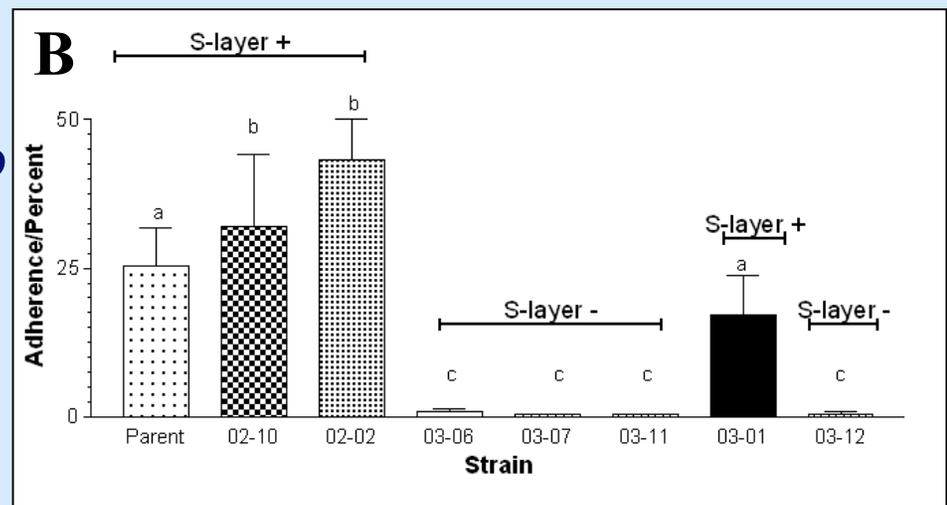
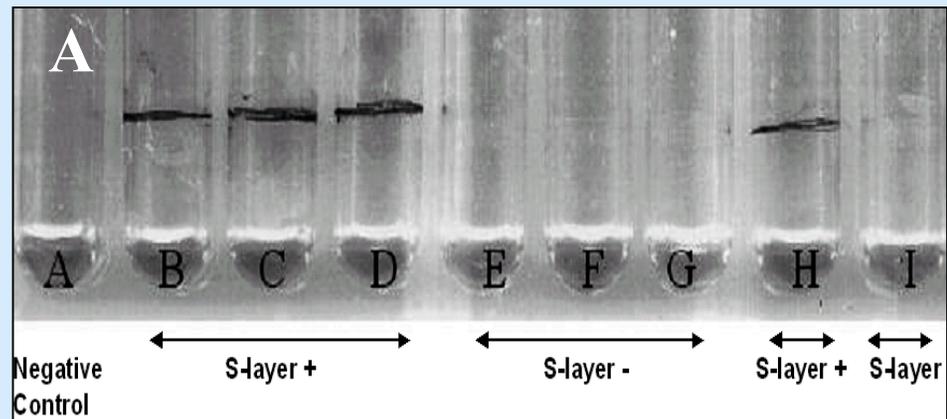
## A) Biofilm

–Minimal media on glass

## B) Adherence to cells

–CHSE 214

- S-layer major adhesin *in vitro*  
–Obscures any effect of pili



# Attenuated virulence of pili mutant strains

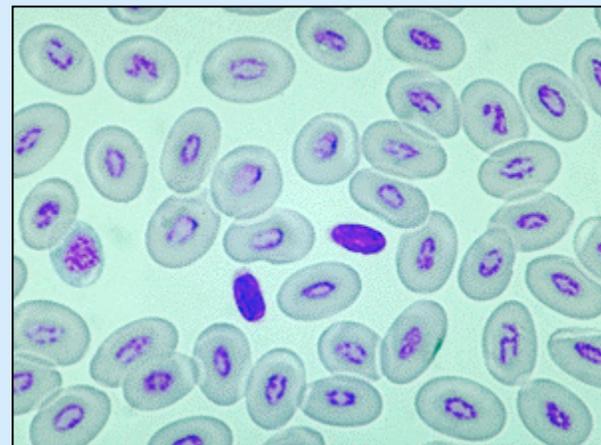
- **Injection**
  - All three strains behave as wild type
- **Immersion**
  - **Morbidity**
    - **Significantly reduced in TapA deficient strains**
  - **Mean time to death (MTTD)**
    - **Increased in FlpA deficient strain**
- **Pili required for adherence or invasion**
  - “Restoration” of virulence on by immersion

Bacterial Strain	Salmon Stock	<u>Immersion</u>	MTTD/days	Hazard ratio
		Cumulative Percent Mortality		
A449 (wt)	St. John	39.2% (38/97)	12	/
Saline	St. John	0% (0/90) *	/	/
<i>flpA</i> ::ko	St. John	28.7% (27/94)	17	1.7
<i>tapA</i> ::ko	St. John	25.3% (21/83) *	13	1.4
<i>tapA</i> ::ko/ <i>flpA</i> ::ko	N/D	N/D	N/D	N/D
A449 (wt)	Sackville	90.0% (72/80)	5	/
Saline	Sackville	0% (0/80) *	/	/
<i>tapA</i> ::ko/ <i>flpA</i> ::ko	Sackville	76.3% (60/80) *	10	1.45

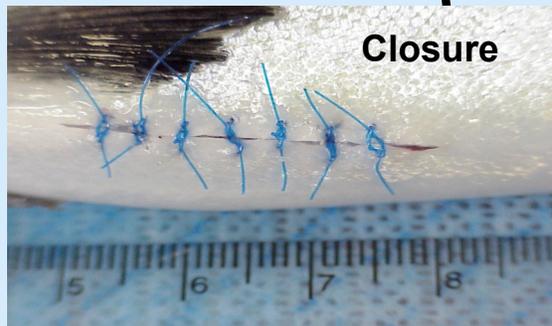
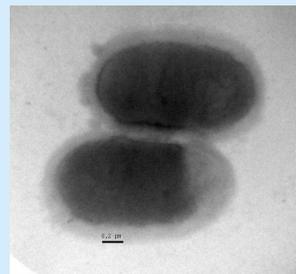
Bacterial Strain	Salmon Stock	<u>Intraperitoneal</u>	MTTD/days	Hazard ratio
		Cumulative Percent Mortality		
A449 (wt)	Sackville	45.0% (18/40)	6.5	/
Saline	Sackville	0% (0/40) *	/	/
<i>flpA</i> ::ko	Sackville	50.0% (19/38)	7.5	1.2
<i>tapA</i> ::ko	Sackville	39.0% (16/41)	7.5	1.2
<i>tapA</i> ::ko/ <i>flpA</i> ::ko	Sackville	37.5% (15/40)	8.0	1.3

# Iron sequestration *In vivo* microbial survival

- *In vivo* levels of free iron below that required for microbial growth
- Host acute phase response includes up-regulation of iron binding proteins
- Iron sequestration strategies important for pathogens

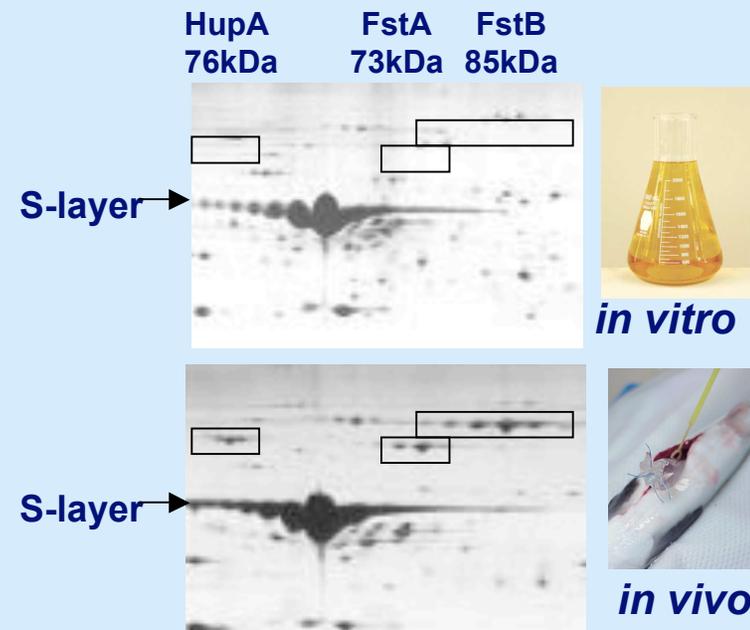


# *In vivo* culture



# Iron scavenging under low iron conditions

- Expression of three OMPs in low iron/*in vivo* growth
  - Siderophore receptors
    - *hupA*
    - *fstB*
    - *fstA*
- Siderophore system
  - High affinity chelators and receptors for Fe and Fe-containing compounds
- Same expression patterns seen in avirulent strains



Adapted from Ebanks RO, Dacanay A, Goguen M, Pinto DM, Ross NW. Proteomics (2004) 4(4);1074

## Attenuated virulence of siderophore receptor mutant strains

- Immersion
  - Morbidity
    - Significantly reduced morbidity in *fstB::AH*
    - *fstA::AH* and *hupA::AH* behave as wt.
  - Mean time to death
    - Increased in *fstA::AH*
- Injection
  - Significantly reduced morbidity in  $\Delta$ *fstB*.
  - *fstA::AH* and *hupA::AH* behave as wt.
  - *fstB* required for virulence
  - Adverse effect on microbial growth vs. pathophysiological effect

Bacterial Strain	Immersion	
	Cumulative Percent Mortality	Median survival
A449	90% (72/80)	6
Saline	0% (0/40)*	Undefined
<i>fstA::AH</i>	82.5% (33/40)	15
<i>fstB::AH</i>	2.5% (1/40)*	Undefined
$\Delta$ <i>fstB</i>	N/D	N/D
<i>hupA::AH</i>	60.0% (24/40)	

Bacterial Strain	Intraperitoneal	
	Cumulative Percent Mortality	Median survival
A449	89.1% (41/46)	6
Saline	0% (0/46)*	Undefined
<i>fstA::AH</i>	84.8% (39/46)	8
<i>fstB::AH</i>	N/D†	N/D
$\Delta$ <i>fstB</i>	10.4% (5/46)*	Undefined
<i>hupA::AH</i>	93.8% (45/48)	5

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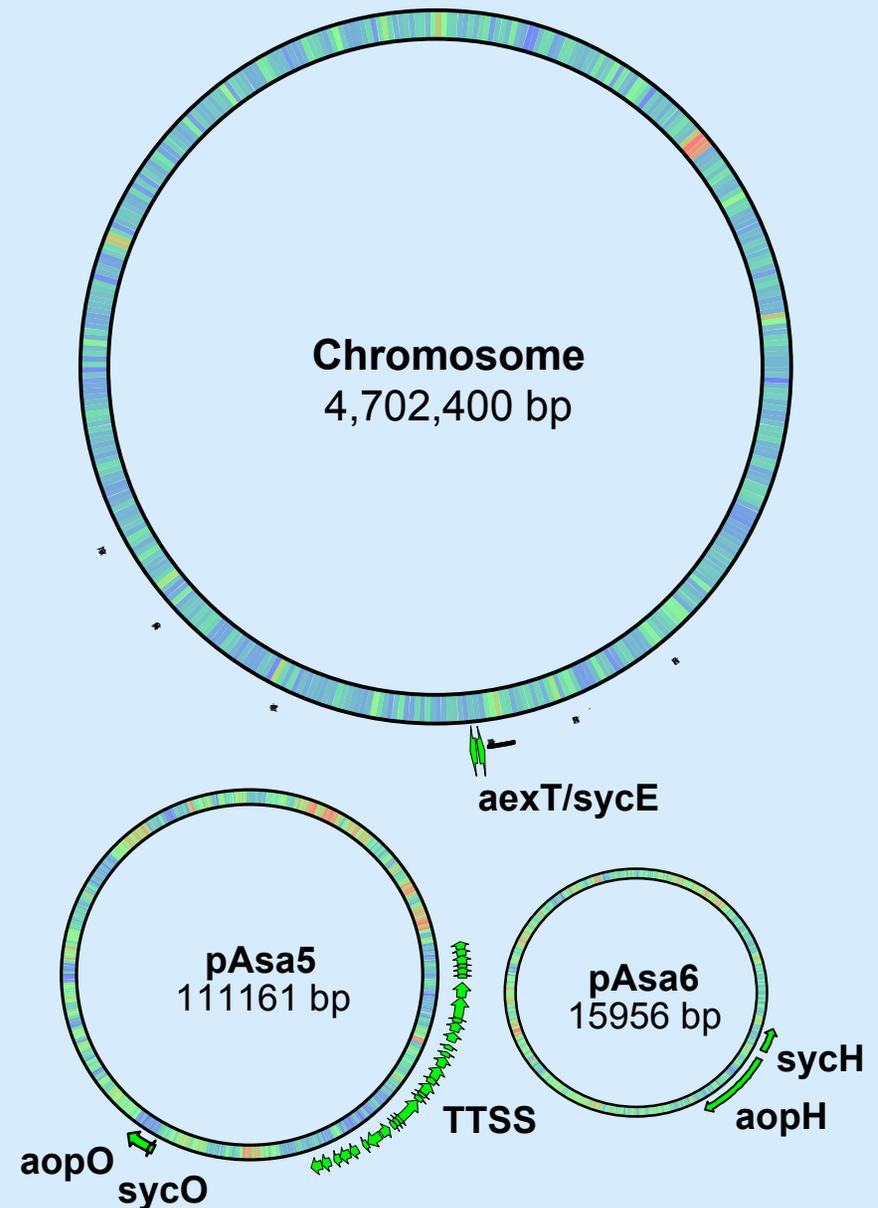
**Host Invasion**

**Overt Disease**



# Type III secretion

- **Delivery system for virulence factors into host-cells to modulate immune function**
- **Consists of**
  - **Secretory apparatus (pores)**
    - IM, OM and host
  - **Effectors**
- **Downstream effects**
  - **Phagocytosis**
    - Antimicrobial release
  - **Cytokine release**
- **The *A. salmonicida* TTSS is not consolidated on e.g. one plasmid**



# Variable virulence of TTS mutant strains

- **OM Pore**
  - Injection, immersions
    - No morbidity in  $\Delta ascC$
  - Effectors
    - Injection, immersions
      - All three effector knockout strains behave as wild type
- **Effectors**
  - Injection
    - All three effector knockout strains behave as wild type
  - Immersion
    - Significantly reduced morbidity in  $\Delta aopH$ ;  $\Delta aexT$  and  $\Delta aopO$  behave as wt.
    - Increased Mean time to Death in  $\Delta aopH$  and  $\Delta aexT$

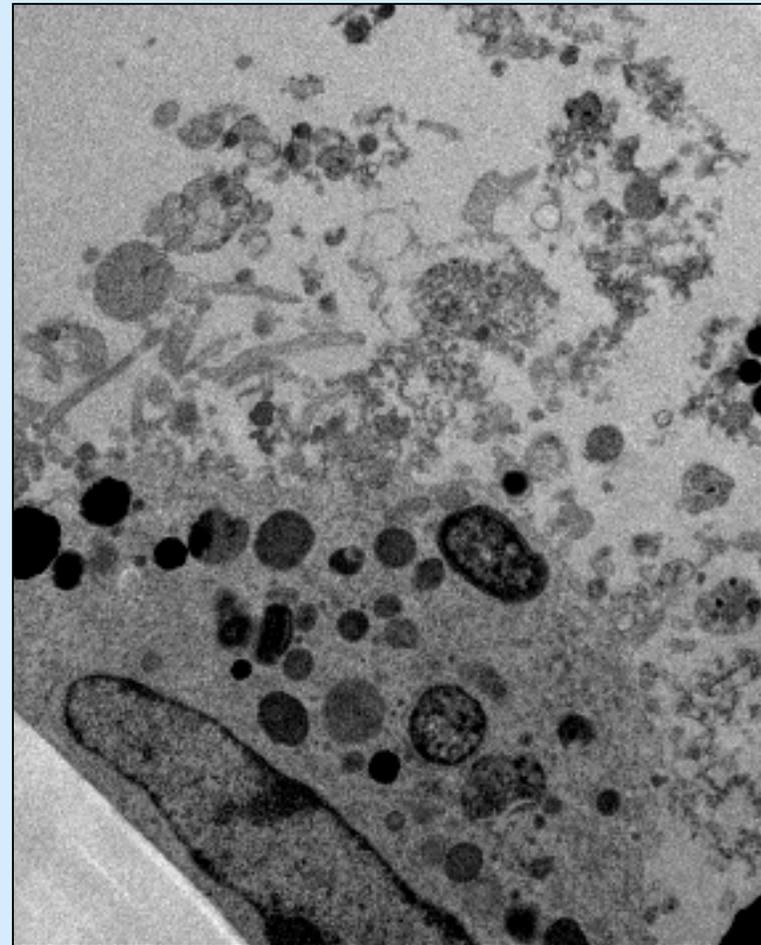
Strain	Immersion	
	Cumulative morbidity	Median survival
A449	60.0% (48/80) <sup>a</sup>	27
PBS	0% (0/80) <sup>b*</sup>	Not defined
$\Delta ascC$	0% (0/80) <sup>b*</sup>	Not defined
$\Delta aexT$	47.5% (38/80) <sup>a</sup>	39
$\Delta aopH$	35.0% (28/80) <sup>c*</sup>	49
$\Delta aopO$	52.5% (42/80) <sup>a</sup>	29

Strain	Intraperitoneal	
	Cumulative morbidity	Median survival
A449	76.3% (45/59) <sup>e</sup>	5
PBS	0% (0/62) <sup>f*</sup>	Not defined
$\Delta ascC$	0% (0/60) <sup>f*</sup>	Not defined
$\Delta aexT$	71.0% (44/62) <sup>e</sup>	5
$\Delta aopH$	67.2 % (41/61) <sup>e</sup>	5
$\Delta aopO$	84.9% (56/66) <sup>e</sup>	5

# Furunculosis aetiology

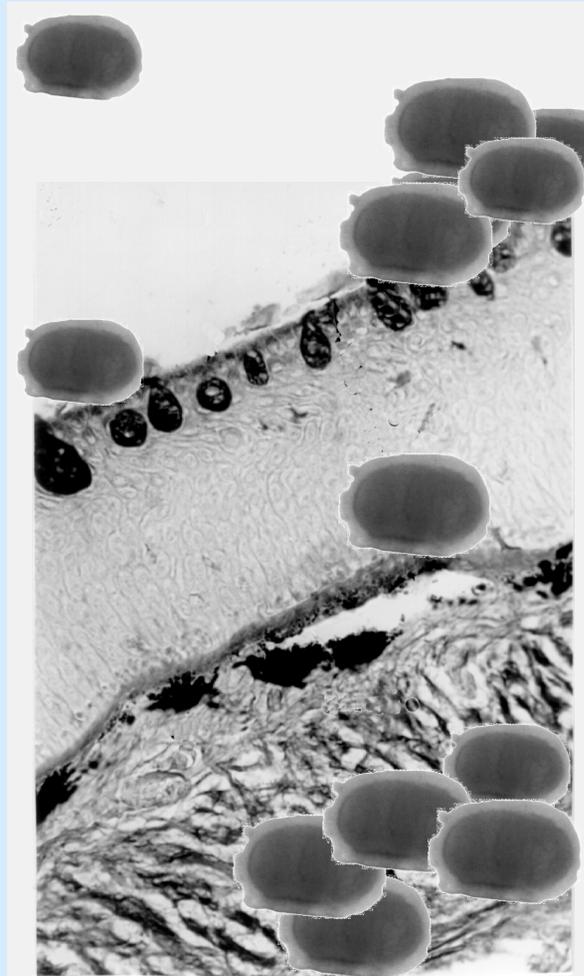
- $\Delta ascC$  data shows TTSS is required for virulence
  - Avirulent by immersion and injection
- Any single effector not absolutely required
  - *aexT* & *aopO*
    - Shared targets?
  - *aopH*
    - Required for invasion?
- $\Delta ascC$  data also show TTSS is required after invasion
  - Secondary invasive step
    - Facultatively intracellular?



# Summary

**Environment**  
**S-layer (*vapA*)**

**Adherence**  
**Pili (*tapA*, *flpA*)**  
**S-layer (*vapA*)**



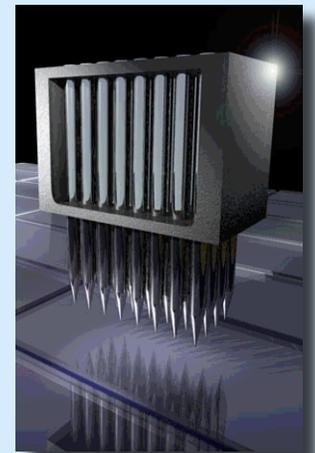
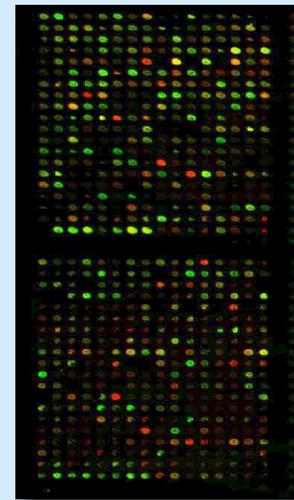
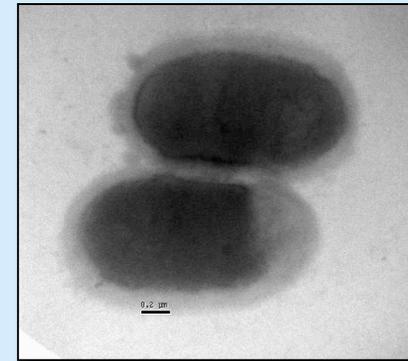
**Colonisation**  
**S-layer (*vapA*)**  
**Pili (*tapA*, *flpA*)**  
**TTS (*ascC*)**

**Invasion**  
**Pili (*tapA*, *flpA*)**  
**S-layer (*vapA*)**  
**TTS (*ascC*, *aopH*)**  
**Oxidative Stress (*sodA*)**  
**TTS (*aopE*, *aopO*)**

**Overt Clinical Disease**  
**TTS (*ascC*, *aopE*)**  
**Oxidative Stress (*sodA*)**  
**Iron Sequestration (*fstB*)**  
**Iron Sequestration (*fstA*, *hupA*)**

# Using microarrays for comparative genomics

- Which genes are present in all virulent strains?
- Is there a correlation between virulence and gene diversity?
- Are these genes expressed during infection?
- We have a myriad of variable markers to use
- The data is obtained at once: ability to "resample" the data depending on the problem
- Based on coding potential = real biology (not arbitrary determinants)



# **A.sal microarray design**

## **Amplicons: PCR-amplified genomic DNA**

- 2128 primer pairs to be selected
  - Represent ORFs selected
  - Primers internal to the gene
  - 21-mers
  - Minimum amplicon size = 160 nt
  - Minimum ORF = 160 nt
  - Primers must work under uniform PCR conditions
  - No false priming

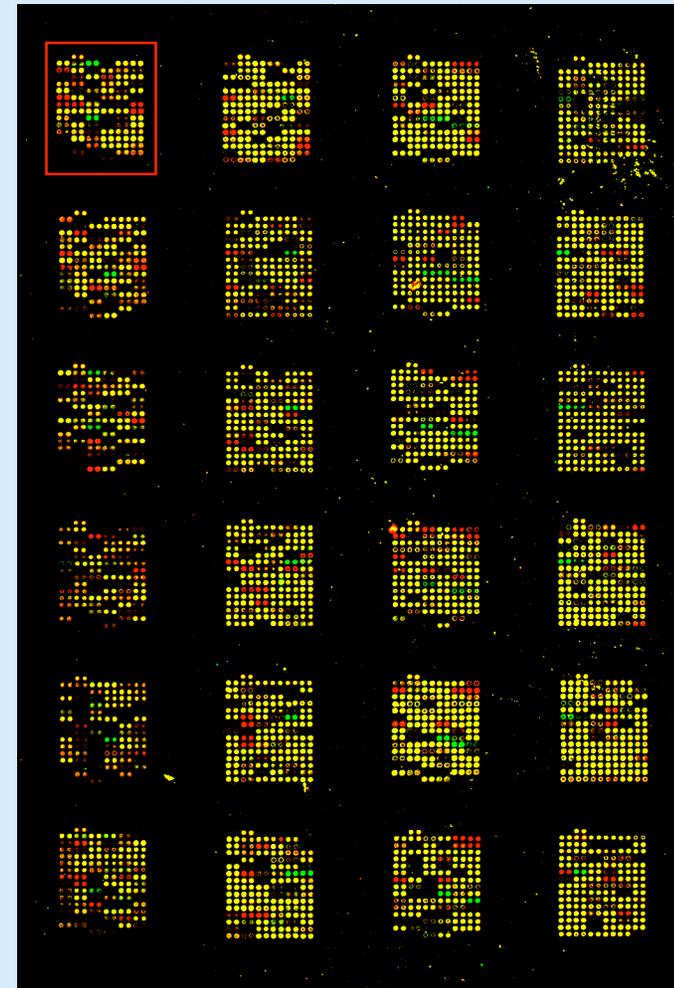
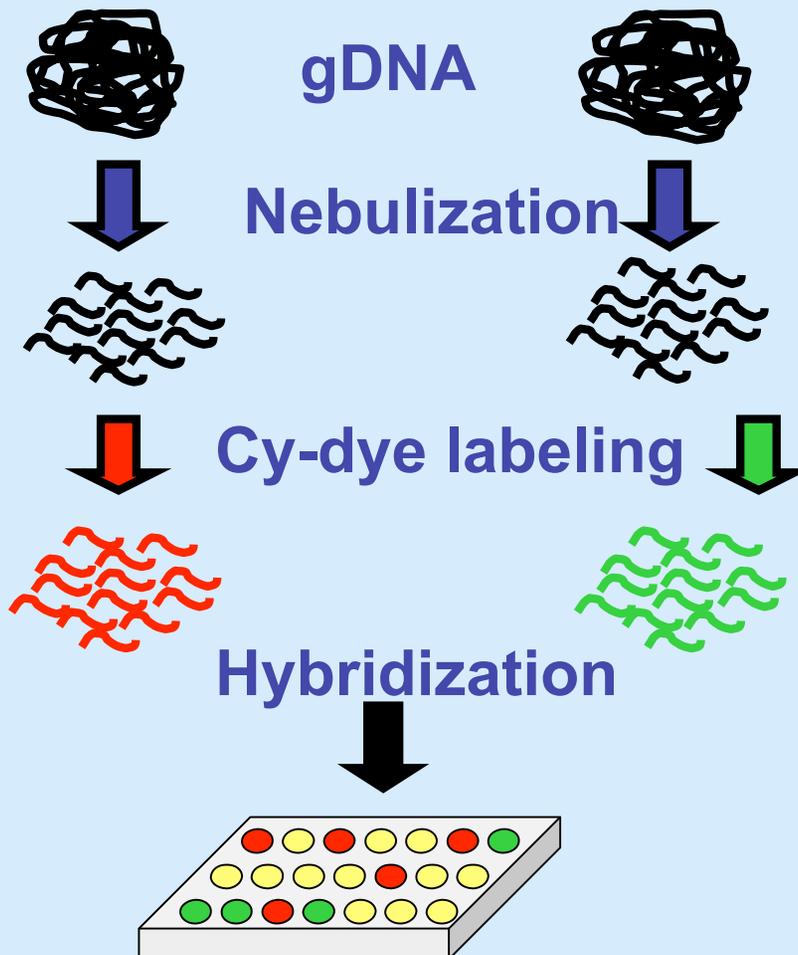
### **GENE FUNCTIONS:**

- Outer membrane proteins
- Flagella/pili
- Secretion systems
- Toxins
- Iron-restriction proteins
- Surface carbohydrates
- Antibiotic resistance
- Plasmids
- Sigma factors
- Transport proteins
- Other virulence factors

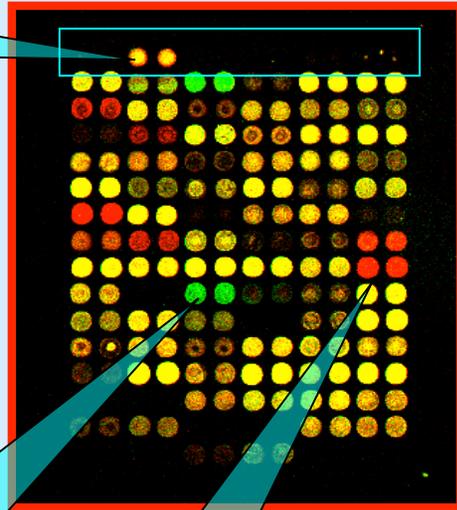
# Microarray CGH

Asal A449

Test strain



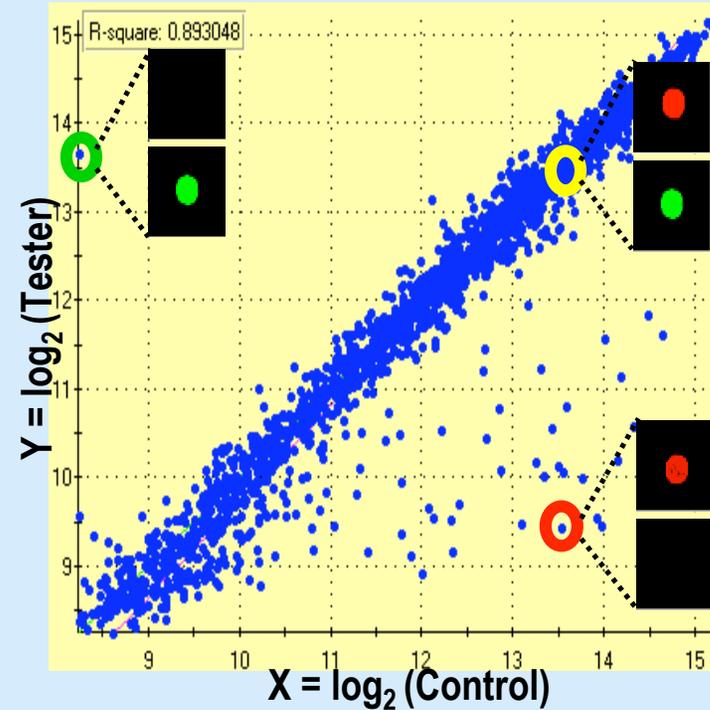
**Controls**



**Duplicated**

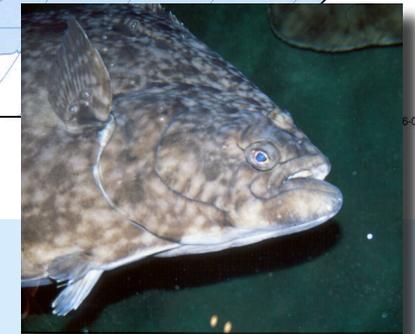
**Divergent  
or Absent**

# Interpreting CGH data



-  Higher in Tester
-  Equal in both
-  Higher in Asal  
A449

- *Aeromonas bestiarum*
- *A. caviae*
- *A. salmonicida achromogenes*
- *A. salmonicida masoucida*
- *A. salmonicida salmonicida*
- *A. salmonicida atypical*



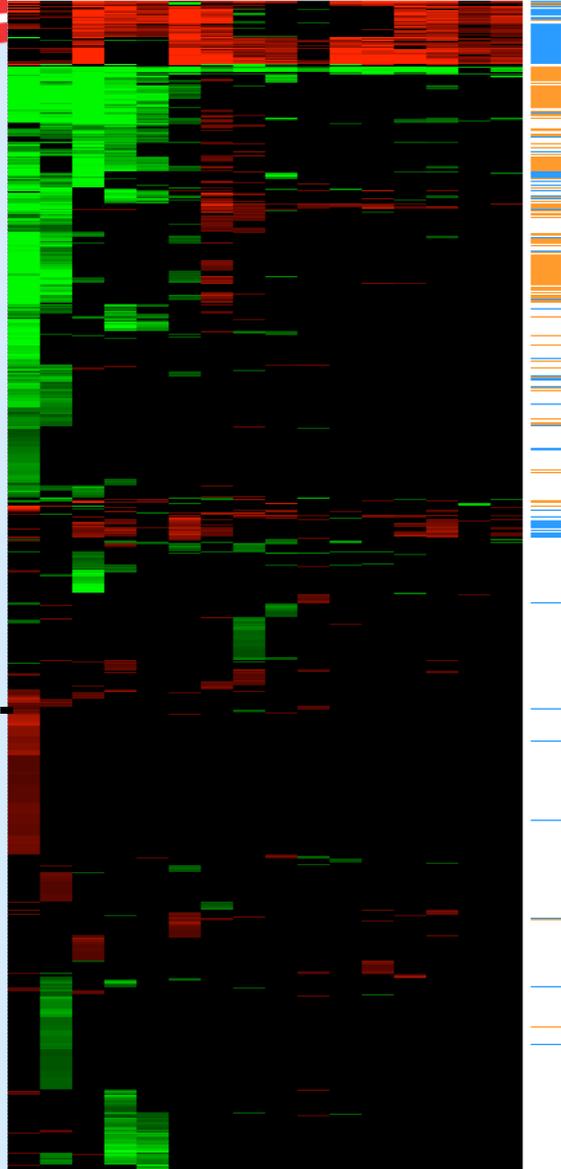
a

*A. bestiarum*  
*A. caviae*  
masoucida  
achromogenes  
N2517  
N2461  
51413  
IMB97-13  
N3395  
BC6129  
33658  
N2660  
IMB97-9  
N1718  
N4715  
N4399

**A**

All genes  
divergent  
in at least  
one strain

- Transposon-associated
- Plasmid-associated



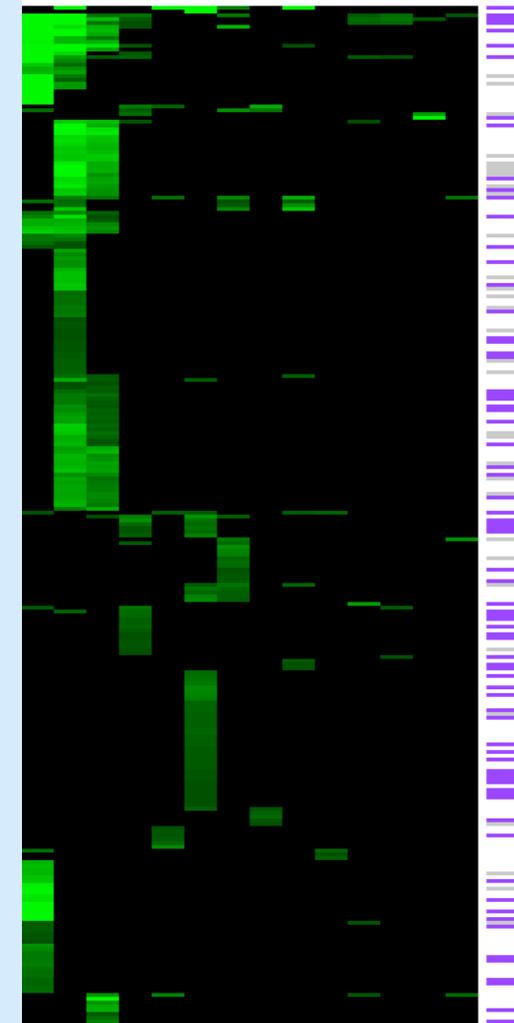
# Divergent genes

**B**

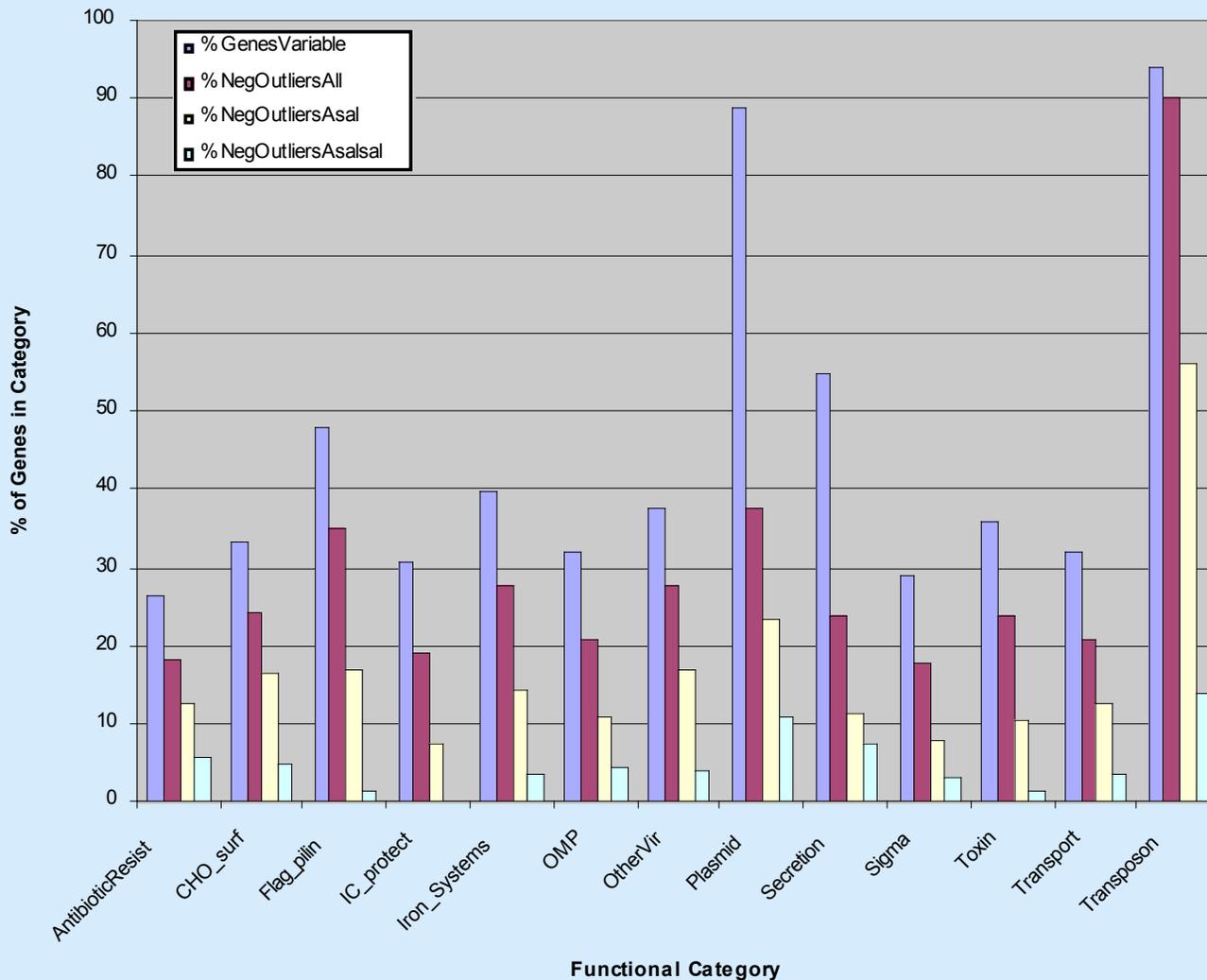
Mobile  
elements  
removed

- OMP
- Pilin

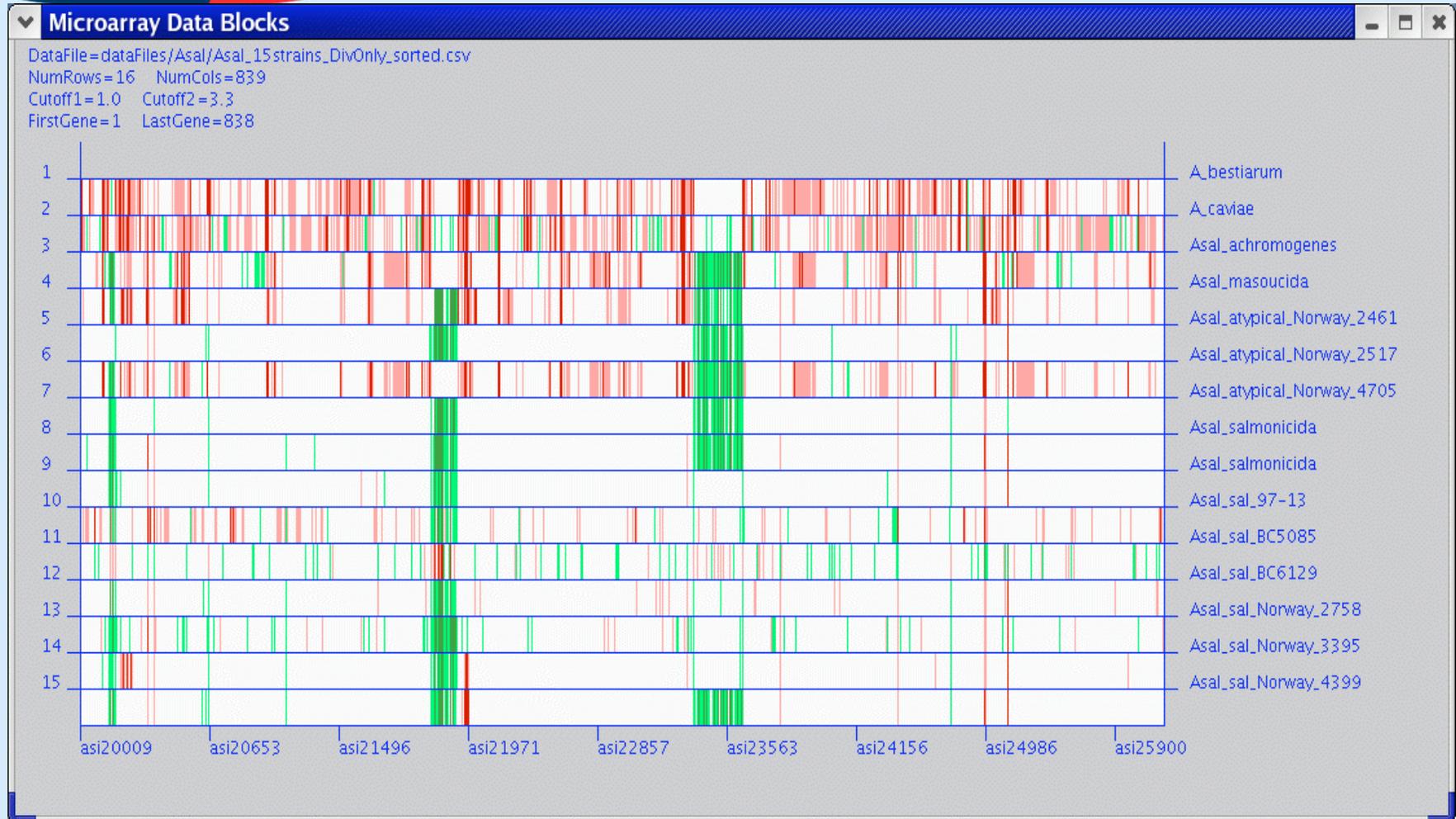
masoucida  
achromogenes  
N2517  
N2461  
51413  
IMB97-13  
N3395  
BC6129  
33658  
N2660  
IMB97-9  
N1718  
N4715  
N4399



# Divergence by function



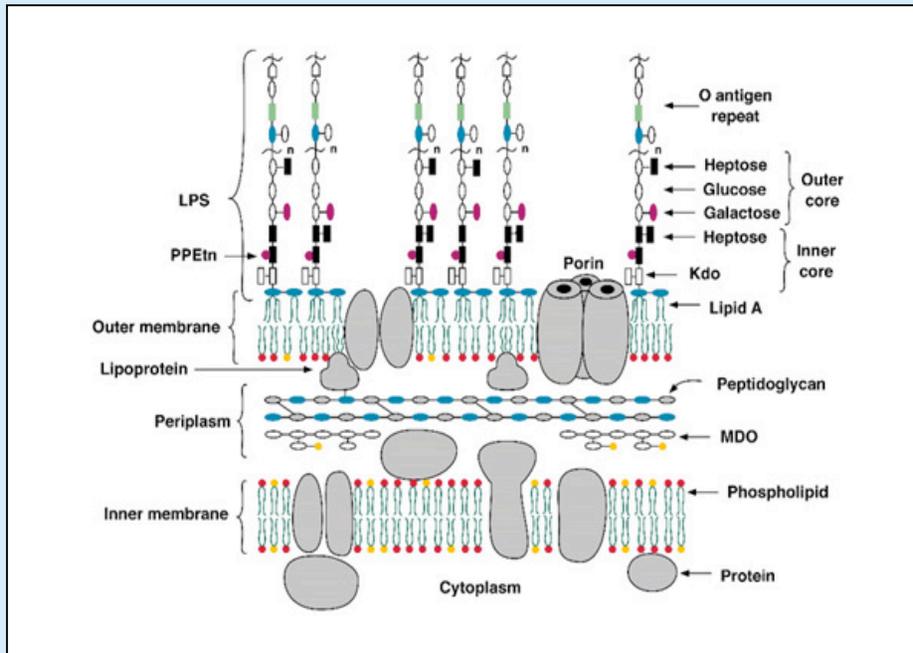
# Divergent and conserved genes – Asal typing



# Summary

- **DNA microarrays and comparative genomic hybridizations (CGH) can be used for strain typing.**
- **Divergent genes appear to be on the mobile elements: plasmids and transposons**
- **Conserved and duplicated genes are appropriate targets for vaccine development. DNA microarrays and CGH are efficient tools for preliminary target identification.**
- **Flagellar, pilin, Type III SS genes may be appropriate targets.**
- **There is no apparent relationship between the number of divergent genes and host species or geographical origin, although it must be noted that there are few Function Unknown (FUN) genes on this array.**

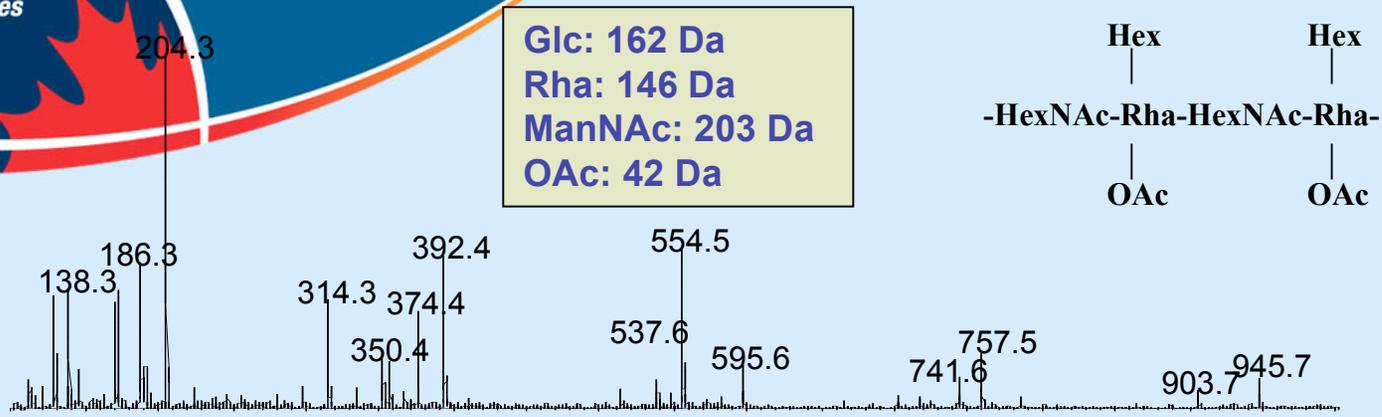
# Structural analysis of LPS of *A.sal* by microscale CE-MS analysis



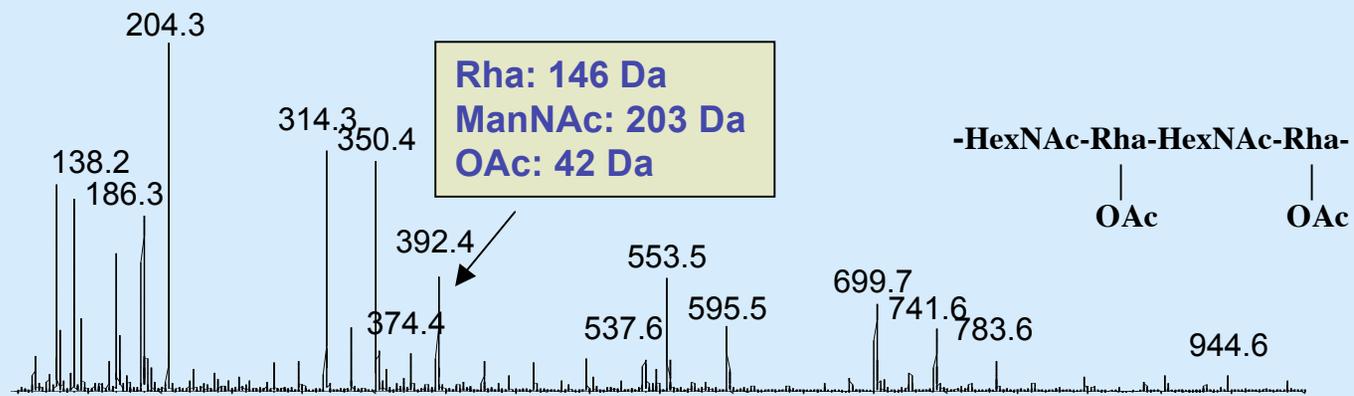
- Physical integrity and functioning of bacterial cell membrane
- Assembly and maintenance of A-layer
- Mediates host cell invasion
- Responsible for serological specificity

# *A. salmonicida* isolates

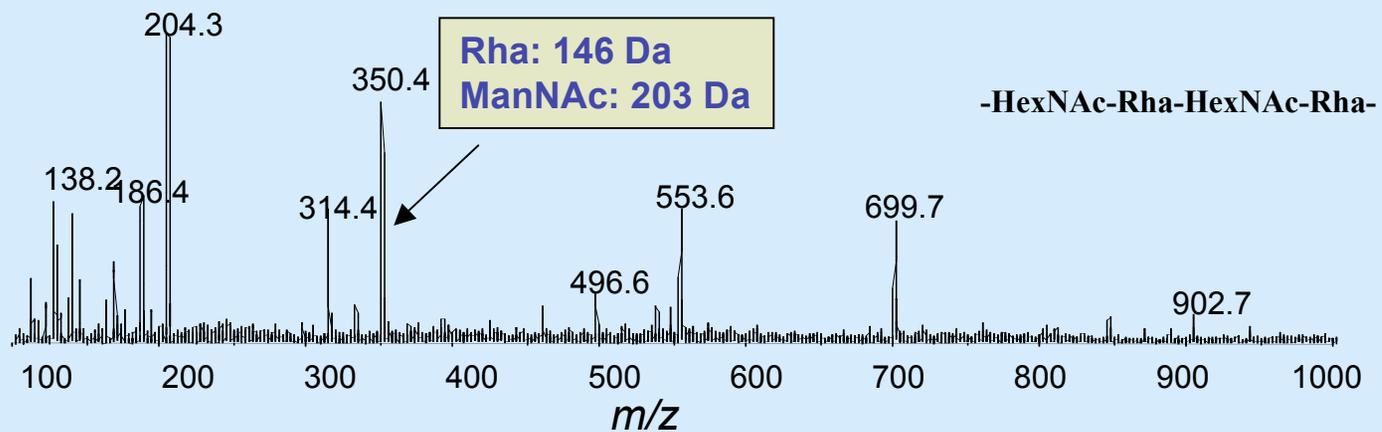
**Strains:**  
BC6129  
BC5085  
N3395  
33658  
N4399  
N2461  
N2758



**Strain:**  
N4705



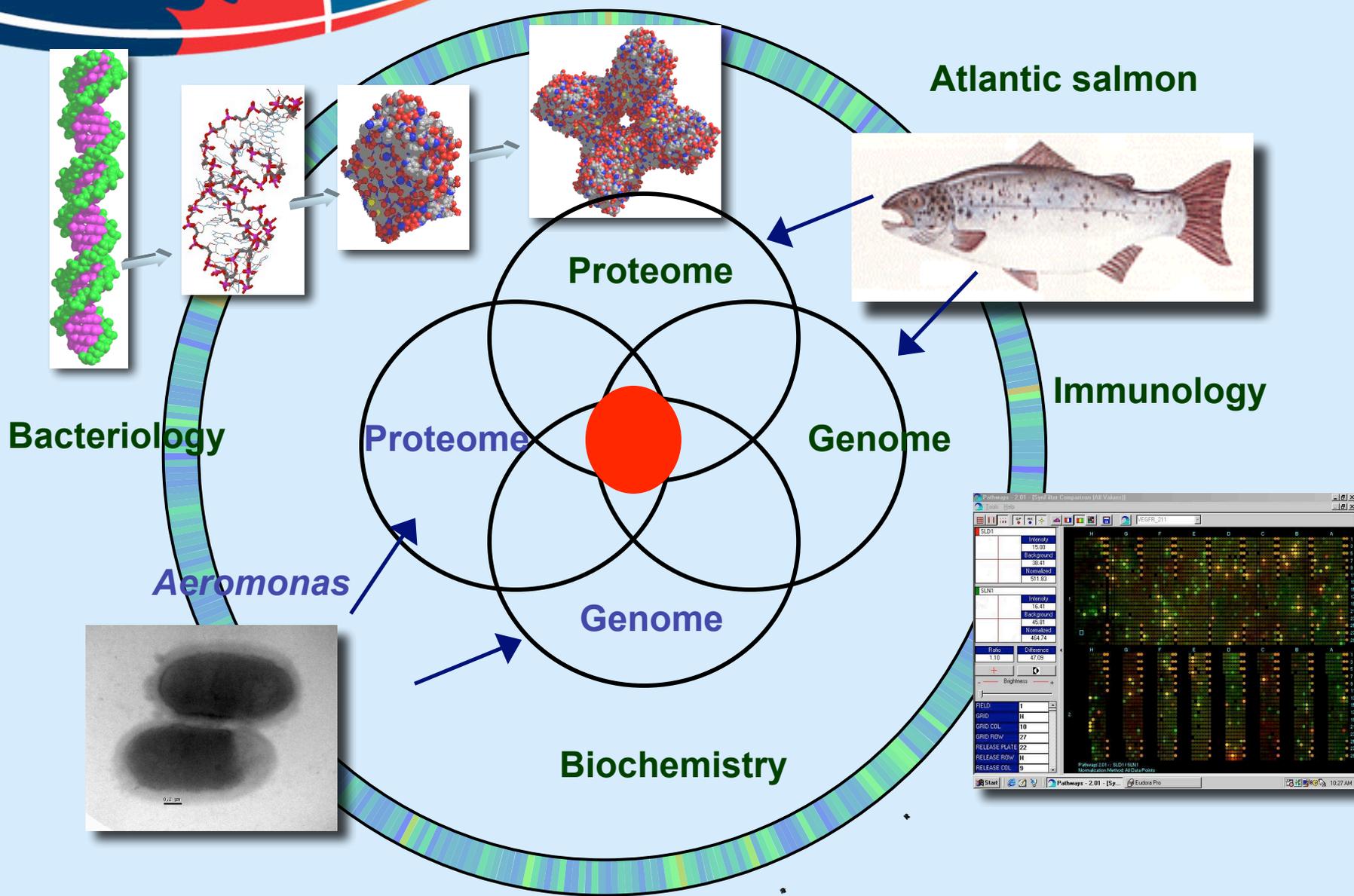
**Strains:**  
N2517  
33659



# Summary

- **CE- MS-based microscale method for analysis of polysaccharides was developed and applied to the analysis of *A. salmonicida* isolates.**
- **Most of *A. salmonicida* isolates produce a complete O-chain polysaccharide.**
- ***A. salmonicida* strain N4705 was shown to produce an incomplete O-chain lacking a terminal glucose residue, while strains 33659 and N2517 were shown to produce an incomplete O-chain lacking both an O-acetyl group and a terminal glucose residue.**

# Overall Summary



## **Next steps**

- **Genome annotation**
- **Continue challenge experiments**
- **Continue characterizing host immune response**
- **Started construction of the full genome array – CGH and transcript profiling**
- **Vaccine and vaccine delivery development**

# The Team

## Management

**Laura Brown**

Gary Fudge

## Module 1

### **Identifying Target Antigens**

*John Nash*

Dev Pinto

Laura Brown

Eleonora Altman

Jianjun Li

## Module 2

### **Vaccine Delivery Systems**

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*Vanya Ewart*

(Neil Mattatall)

Jess Boyd

Kelly Soanes

## Module 0

### **Vaccine testing and challenge**

*Andrew Dacanay*

## Module 3

### **Host Response**

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Neil Ross

John Walter

Evelyn Soo

Luis Afonso

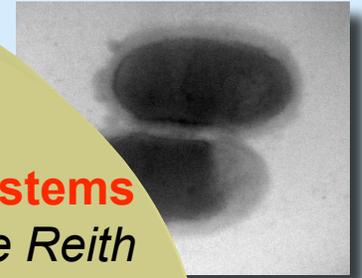
Rafael Garduno

Andrew Dacanay

## Business

## Development

*Denise Lalanne*



# Acknowledgements

## IMB

### **Tony Manning**

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Dave Craft  
Steve Avery  
Steve Locke  
Jeremy Melanson  
Bob Richards

JC Achenbach

### **Debbie Plouffe**

### **Sathish Prasad**

### **Kirty Solanky**

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Darren Sarty  
Pauline Murphy

### **Mark Fast**

Jane Osborne  
Russell Easy

## IMB

Joseph Hui  
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Ken Chisholm  
Steve Locke  
Janet Munholland  
Hannah McKenzie  
Leah Knickle

## IBS

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Simon Foote  
Wendy Findlay  
Jacek Stupak

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### **Zhang Wang**

Vandana Chandan  
Scott Ferguson  
Blair Harrison

## IMB

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Cynthia Stone  
David Barrington  
Joanne Burbidge  
Brent Higgins  
Angie Lewis  
Byron Parsons  
Gary Simpson  
Heidi Verheul  
Jennifer Kimball  
Jennifer Heal  
Allison Maffey  
Bruce Curtis  
Emmanuel  
Egbosimba

## BRI and GHI

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### **Dr. André Nantel (BRI)**

Tracy Rigby (BRI)

### Dalhousie U.

Elizabeth Garduño  
Jaime Vickers  
John Batt  
Jerry Whynot  
Steve Fletcher  
Don Lawrence

## Donors

Dr. Duncan Colquhoun, *National Vet. Institute, Norway*

Dr. William Kay, *University of Victoria*

Dr. Joanne Constantine, *BC Ministry of Agriculture, Food and Fisheries*

Dr. Gilles Olivier, *Fisheries and Oceans Canada*

Dr. Vera Lund, *Fiskeriforskning, Norway*

Dr. Rocco Cipriano, *USGS, WV, USA*

Dr. Mark Strom, *NOAA*

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